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Nosema chrysorrhoeae n. sp. (Microsporidia), isolated from browntail moth (*Euproctis chrysorrhoea* L.) (Lepidoptera, Lymantriidae) in Bulgaria: Characterization and phylogenetic relationships

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Abstract

A new microsporidian parasite *Nosema chrysorrhoeae* n. sp., isolated in Bulgaria from the browntail moth (*Euproctis chrysorrhoea* L.), is described. Its life cycle includes two sequential developmental cycles that are similar to the general developmental cycles of the *Nosema*-like microsporidia and are indistinguishable from those of two *Nosema* spp. from *Lymantria dispar*. The primary cycle takes place in the midgut tissues and produces binucleate primary spores. The secondary developmental cycle takes place exclusively in the silk glands and produces binucleate environmental spores. *N. chrysorrhoeae* is specific to the browntail moth. Phylogenetic analysis based on the ssu rRNA gene sequence places *N. chrysorrhoeae* in the *Nosema*/*Vairimorpha* clade, with the microsporidia from lymantriid and hymenopteran hosts. Partial sequences of the lsu rRNA gene and ITS of related species *Nosema kovacevici* (Purrini K., Weiser J., 1975. Natürliche Feinde des Goldafters, *Euproctis chrysorrhoea* L., im Gebiet von Kosovo, FSR Jugoslawien. Anzeiger fuer Schädlingskunde, Pflanzen-Umweltschutz, 48, 11–12), *Nosema serbica* Weiser, 1963 and *Nosema* sp. from *Lymantria monacha* was obtained and compared with *N. chrysorrhoeae*. The molecular data indicate the necessity of future taxonomic reevaluation of the genera *Nosema* and *Vairimorpha*.

Keywords: Microsporidia; *Nosema chrysorrhoeae*; *Nosema kovacevici*; *Nosema serbica*; Ultrastructure; Taxonomy; rDNA phylogeny; *Euproctis chrysorrhoea*

1. Introduction

The browntail moth (*Euproctis chrysorrhoea* L.) occurs in Central and Eastern Europe in periodic local outbreaks. It is a serious pest of orchards and broadleaf forests in these regions. *E. chrysorrhoea* also elicits contact dermatitis and strong allergic reactions in some humans. Although occasionally outbreaks are reduced by nuclear polyhedrosis virus and pathogenic fungi, microsporidia are important regulators of the population dynamics of *E. chrysorrhoea* (Sterling and Speight, 1989).

Several microsporidian species have been described from the browntail moth. *Endoreticulatus* (formerly *Pleistophora*) *schubergi* was described by Zwölfer in 1927. Later, Lipa (1964) recovered a *Nosema* sp. in a browntail moth population in Poland, and Sidor et al. (1975) found *Nosema* sp. infections in Macedonia. Purrini and Weiser (1975) described

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Nosema kovacevici from *E. chrysorrhoea* in Kosovo in former Yugoslavia. No data on ultrastructure, primary spore cycle were provided in these publications and rDNA sequencing was not available.

In Bulgaria, there have been several reports of microsporidian infections in *E. chrysorrhoea* (Pilarska et al., 2001, 2002; Solter et al., 2000). These authors recovered several microsporidian isolates and conducted light and transmission electron microscopy studies, (Pilarska et al., 2001, 2002), however, no species descriptions were published. This paper describes a microsporidium found in an *E. chrysorrhoea* population in Central Bulgaria.

2. Materials and methods

2.1. Origin of isolate, comparative isolates, and laboratory hosts

The microsporidium was recovered from *E. chrysorrhoea* larvae collected in 2001 from *Quercus cerris* L. in the vicinity of Karlovo, Central Bulgaria. Three of 40 collected larvae were infected; the species description is based on material obtained from one of these larvae. Infected silk glands were homogenized in a glass tissue grinder in distilled water, the spore suspension was washed twice by successive centrifugation in distilled water and the cleaned spores used for infection experiments.

Morphological comparisons of *N. kovacevici* (Purrini and Weiser, 1975), *Nosema serbica* Weiser, 1963, and *Nosema* sp. from *Lymantria monacha* were made from syntype slides in the collection of Jaroslav Weiser, deposited in the Natural History Museum in Vienna, Austria.

Eggs of *E. chrysorrhoea* L. and larvae of the gypsy moth, *Lymantria dispar* L. were obtained from BIOLA, Chelčice, Czech Republic. Larvae of *Mamestra brassicae* L. and *Spodoptera littoralis* (Boisd.) were obtained from the laboratory colony maintained at the Institute of Crop Production, Prague, Czech Republic.

The GenBank accession numbers of sequences for *N. chrysorrhoeae*, *N. serbica*, *N. kovacevici* and *Nosema* sp. from *L. monacha* and of other microsporidian species used for phylogenetic analysis are the following: *Amblyospora californica* (U68473), *Amblyospora cinerei* (AY090057), *Amblyospora* sp. (*A. salinarius*) (L28960), *Amblyospora stimuli* (AF027685), *Amblyospora weiseri* (AY090048), *Ameson michaelis* (L20293), *Brachiola algerae* (AF069063), *Brachiola algerae* (AF069063), *Brachiola algerae* (AY230191), *Brachiola algerae* (L28961), *Bryonosema plumatellae* (AF484690), *Encephalitozoon cuniculi* (AJ005581), *Glugea anomala* (AF044391), *Gurleya daphniae* (AF439320), *Gurleya vavrai* (AF394526), *Heterosporis anguillarum* (AF387331), *Larssonia obtusa* (AF394527), *Loma embiotocia* (AF320310), *Nosema apis* (U26534), *Nosema apis* (U97150), *Nosema bombi* (AY008373), *Nosema bombycis* (AY259631), *Nosema bombycis* (L39111), *Nosema carpocapsae* (AF426104), *Nosema ceranae* (U26533), *Nosema disstriae* (L28963), *Nosema epilachnae* (L28964), *Nosema furnacalis* (U26532), *Nosema granulosis* (AJ011833), ***Nosema chrysorrhoeae (AY940656), Nosema chrysorrhoeae (AY940657), Nosema kovacevici (AY940658)***, *Nosema oulemae* (U27359), *Nosema portugal* (AF033316), ***Nosema serbica (AY940659), Nosema sp. (L. monacha) (AY940660)***, *Nosema spodopterae* (AY211390), *Nosema tyriae* (AJ012606), *Nosema vespula* (U11047), *Nucleospora salmonis* (U78176), *Ordospora colligata* (AF394529), *Parathelohania anophelis* (L28969), *Pleistophora typicalis* (AF044387), *Pseudoloma neurophilia* (AF322654), *Pseudonosema cristatellae* (AF484694), *Tubulinosema kingi* (L28966), *Vairimorpha ephestiae* (L28972), *Vairimorpha heterosporum* (L28973), *Vairimorpha cheracis* (AF327408), *Vairimorpha imperfecta* (isolate 1.) (AJ131645), *Vairimorpha imperfecta* (isolate 2.) (AJ131646), *Vairimorpha lymantriae*[1] (AF033315), *Vairimorpha lymantriae* (isolate Leviste) (AF141129), *Vairimorpha lymantriae* (L28974), *Vairimorpha necatrix* (L28975), *Vairimorpha necatrix* (Y00266), *Vairimorpha* sp. (S.richteri) (AF031539), *Vairimorpha* sp. (*P. xylostella*) (AF124331).[2]

2.2. Laboratory infections of E. chrysorrhoea and other lepidopteran larvae

Second instar *E. chrysorrhoea* larvae were infected by spreading spore suspension on *Salix* cf. *babylonica* L. leaves; larvae of the other laboratory hosts were fed small cubes of the artificial diet (Bell et al., 1981) inoculated with spores. Dosages were 10^3–10^4 µl per larva. The larvae were starved for 24 h prior to the treatment and readily consumed the contaminated diet. After treatment, *E. chrysorrhoea* larvae were reared on *Salix* leaves washed with 3% hydrogen peroxide, and other laboratory hosts were reared on artificial diet. All larvae were reared in growth chambers at 24 °C, 16/8 L/D.

2.3. Examination of infected host tissues; transmission electron microscopy

Fresh tissues and tissue smears stained with Giemsa (Sigma Diagnostic Accustain) were examined for the presence of developmental stages and spores using light microscopy. Spores were examined using the agar spore immobilization method (Vávra, 1964), measured with an Image Splitting Eyepiece (Vickers Instruments Ltd) and photographed on a Olympus BX 51 microscope.

For transmission electron microscopy (TEM), infected silk glands were fixed for 24 h in 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4. Fixed tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite.

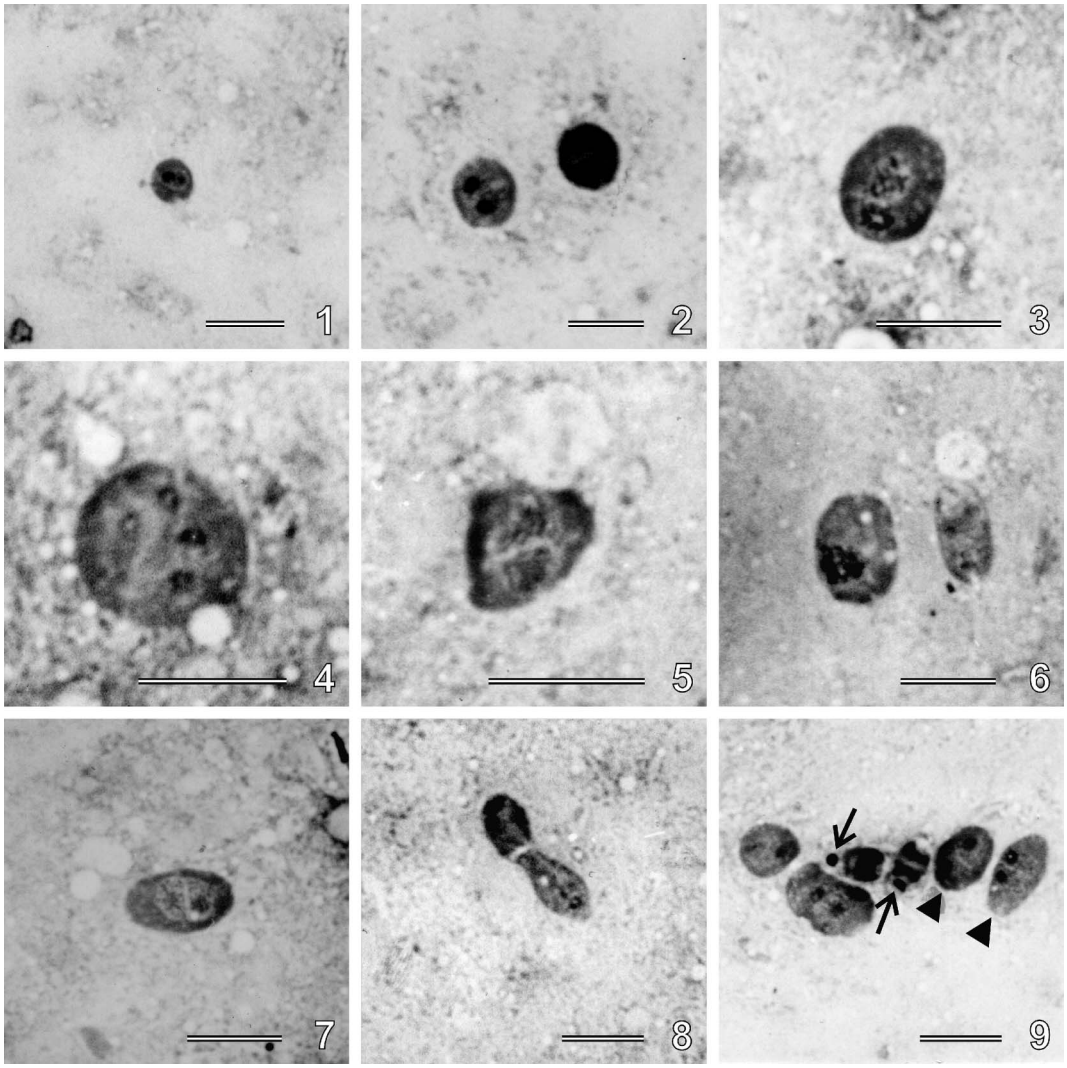
[1] *Vairimorpha lymantriae* is not a valid species name and is being redescribed as *Vairimorpha disparis* (Vávra et al., 2006).

[2] For phylogenetic analysis based on ITS and LSU rRNA gene sequences *Vairimorpha lymantriae* L28974 (= *Vairimorpha* sp.3 from Baker et al., 1994) was used; other *Vairimorpha lymantriae* isolates: *Vairimorpha* sp. 4 L28976 and *Vairimorpha* sp. 5 L28970 (Baker et al., 1994) and ITS region of Nosema portugal AF033316 (Maddox et al., 1999) were included in comparison of nucleotide differences.

Thin sections were cut on a Reichert–Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined and photographed using a Jeol JEM-1010 electron microscope.

2.4. rDNA sequences, phylogenetic analysis

DNA was isolated from fresh purified spores of *N. chrysorrhoeae*, and from the Giemsa-stained the syntype slides of *N. kovacevici*, *N. serbica* and *Nosema* sp. from *L. monacha* (Hyliš et al., 2005). Material was prepared for DNA isolation using the bead beating method (Baker et al., 1994), followed by the DNeasy Tissue Kit (QIAGEN, Germantown, Maryland, USA) procedure. A set of primers ss530f:ls580r for *N. chrysorrhoeae*, and ss1537f:ls580r for *N. kovacevici*, *N. serbica* and *Nosema* sp. were used to amplify conserved and variable regions of small and large subunit rDNA (ss530f GTG CCA GCA GCC GCG G; ss1537f GAA CCA GCA GCA GGA TCA TAA; ls580r GGT CCG TGT TTC AAG ACG G) (slightly modified primers of Weiss and Vossbrinck, 1999). The PCR reactions (95 °C for 2 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min) were conducted in a total volume of 25 μl with 50–100 ng of DNA, 25 pmol of each primer, 1 unit Taq polymerase (TAKARA BIO, Otsu, Shiga, Japan) and buffer/dNTP (TaKaRa) according to manufactured instructions. PCR products were separated using 1% agarose gel electrophoresis, extracted from the gel, purified using the DNeasy Tissue Kit (QIAGEN, Germantown, Maryland, USA), cloned (TOPO TA Cloning Kits, Invitrogen, Carlsbad, California, USA) and sequenced on an automatic



Figs. 1–9. Developmental stages of the primary developmental cycle of *Nosema chrysorrhoeae*, as seen in the Giemsa-stained smears of the midgut epithelial cells of *Euproctis chrysorrhoea*. The total volume of the nuclei is hardly visible, the dark spots are the chromatin. (Bars = 5μm.). Figs. 1 and 2. Early diplokaryotic meronts. Fig. 3. Binucleate meront at the onset of the first nuclear division (there is some asynchrony in the division of the diplokaryon members). Fig. 4. Tetranucleate meront. Fig. 5. Probable early sporont issued from the division of a late tetranucleate meront. Figs. 6 and 7. Late sporonts before the second nuclear division. Fig. 8. Late sporont dividing in two sporoblasts (= disporoblastic sporogony). Fig. 9. Binucleate primary spores show large posterior vacuole and distinctly stained posterosomes (arrows). Late sporonts are at arrowheads.

sequencer (Beckman CEQ 2000 XL). The sequences were aligned using ClustalX program (Thompson et al., 1997), gaps and ambiguously aligned regions were omitted from further analyses. Maximum parsimony (MP) trees were constructed using PAUP 4b10 (Swofford, 2000) with TBR as a branch-swapping method and 1000 bootstrap replicates. Maximum likelihood trees were constructed by PHYML program (Guindon and Gascuel, 2003), using GTR model for nucleotide substitutions with discrete gamma distribution in 8 + 1 categories; all parameters (gamma shape, proportion of invariants) were estimated from dataset. Multiple datasets for ML bootstrap analyses were prepared using SeqBoot (PHYLIP 3.6.3; Felsenstein, 2001). ML bootstrap support was computed in 500 replicates using PHYML program with HKY85 model for nucleotide substitutions and one category of sites with TI/TV ratio estimated from the data set.

3. Results

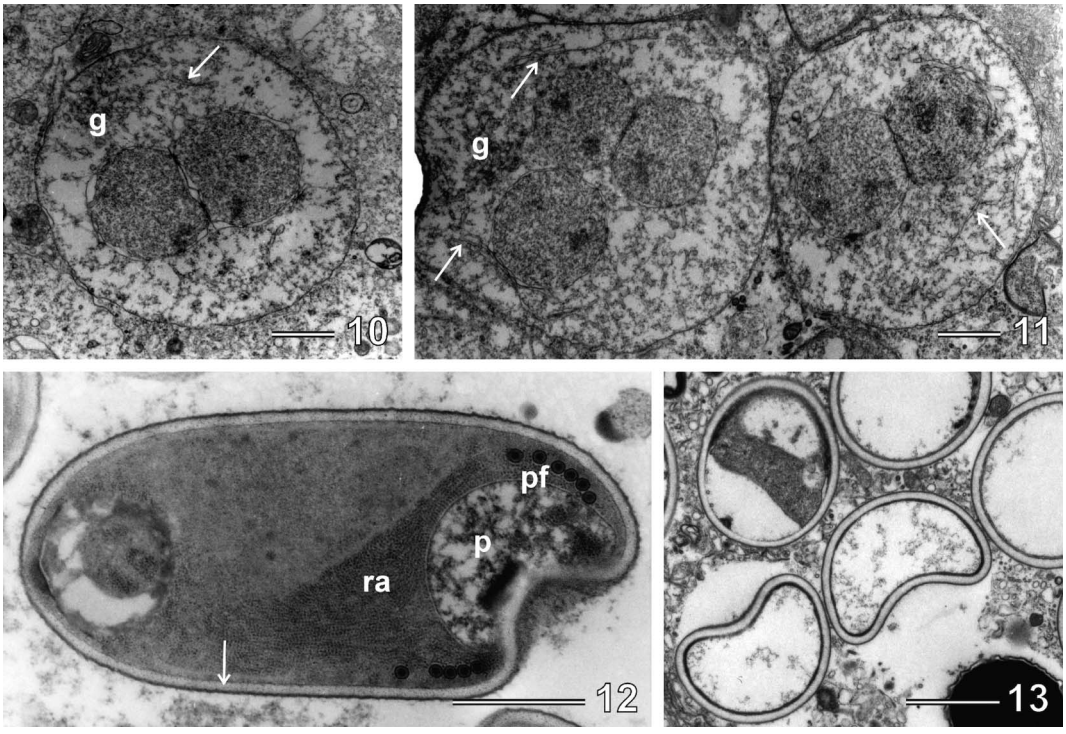
3.1. Life cycle

Our observations demonstrate that the life cycle of *Nosema chrysorrhoeae* consists of primary and secondary developmental cycles, which differ in time and tissue specificity in the host organism and the type of spore produced. Each cycle consists of merogony and sporogony typical of microsporidia in the genus *Nosema*.

3.2. Primary merogonic and sporogonic cycles

Primary merogony and sporogony occur in midgut epithelial cells and longitudinal and circular midgut muscle cells. The first stages observed in Giemsa smears of the midgut tissue were small meronts, with two nuclei in diplokaryotic arrangement (Fig. 1). Later merogonial stages were larger cells with two or four nuclei (Figs. 2–4). Clearly distinct diplokaryotic nuclei were apparent in some Giemsa stained meronts (Fig. 5), but in others the chromatin distribution was such that it was difficult to decide if there was a diplokaryon or if the two nuclei had fused (Fig. 6). Sporonts were elongate and contained two or four nuclei arranged as diplokarya (Figs. 7 and 8). The division of sporonts produced diplokaryotic sporoblasts and immature primary spores (Fig. 9). Mature primary spores occurred in midgut epithelial cells and muscles at about 48 h post inoculation. The primary spores were characterized by large posterior vacuoles. The posterior vacuole and the two nuclei were also visible in Giemsa stained smears (Fig. 9). Stained primary spores measured 4.1 (4.5–5.2) × 1.9 (2.1–2.2) μm ($n = 25$).

At the ultrastructural level, the developmental stages of the primary cycle proved to be difficult to preserve adequately. Meronts, enveloped by a single cytoplasmic membrane containing two or four nuclei arranged as diplokarya, ribosomes, and traces of endoplasmic reticulum were observed (Figs. 10 and 11). The beginning of sporogony was marked by the thickening of the plasmalemma and the for-



Figs. 10–13. *Nosema chrysorrhoeae* primary developmental stages in the midgut epithelial cells of *Euproctis chrysorrhoea* as seen in the electron microscope. Figs. 10 and 11. Two- and tetranucleate meronts with nuclei in the diplokaryotic arrangement. The cytoplasm documents the difficulty in obtaining satisfactory fixation of these stages. Only some ribosomes, traces of Golgi vesicles (g) and of endoplasmic reticulum (arrow) are shown (Bar = 1 μm). Fig. 12. Primary spore showing the large posterior vacuole (p), thin endospore (arrow) and six polar filament coils (pf). Polyribosome aggregates are at ra. (Bar = 1 μm.). Fig. 13. Empty shells of primary spores left after their firing. (Bar = 2 μm.)

mation of more abundant endoplasmic reticulum. The sporoblasts were heavily distorted, extremely electron dense cells with no discernible internal structure (not shown).

Primary spores were relatively well preserved in our material, showing a thin, electron dense, single layer smooth exospore (25–30 nm), a relatively thin, lucent endospore (70–75 nm), and cytoplasm with abundant rows of polyribosomes. The isofilar polar filament was arranged in five to six coils in a single row. The posterior pole of the primary spore contained a vacuole enclosing granular material and was invaginated. Irregular vacuoles probable representing a badly preserved polaroplast were present in the anterior pole of the spore (Fig. 12). Germinated primary spores and spores with posterior vacuole grossly inflated were frequently observed (Fig. 13).

3.3. Secondary merogonic and sporogonic cycles

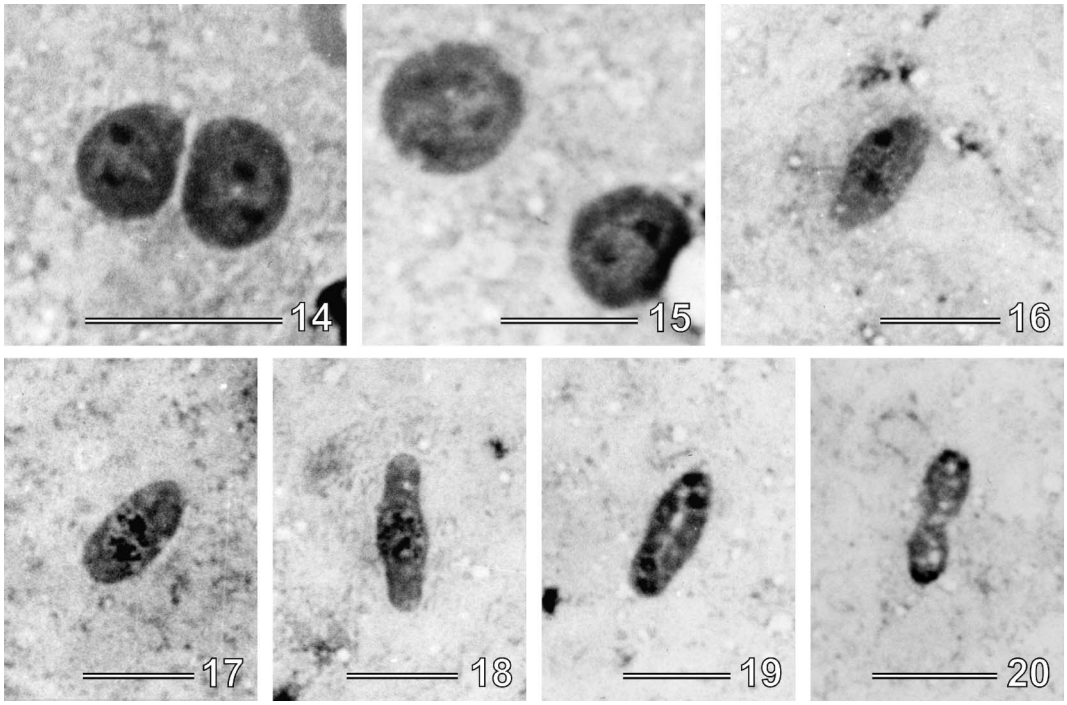
Merogonial and sporogonial stages of the secondary developmental cycle were observed in silk glands 4 days post inoculation (dpi) and were similar in appearance to the stages of the primary cycle. Two or four nuclei arranged as diplokarya were visible in secondary meronts in Giemsa smears (Figs. 14 and 15). Sporonts contained two or four nuclei (Figs. 16–18) and gave rise to two sporoblasts (Figs. 19 and 20). Mature secondary spores were elongate, ellipsoidal to spindle shaped (Figs. 21 and 22), significantly longer than primary spores, and measured 6.12 (5.52–6.67) × 2.21 (1.99–2.38) μm ($n = 50$) when fresh.

Ultrastructurally, the meronts of the secondary developmental cycle did not differ from the meronts of the primary cycle, however, their cytoplasm was more dense and fixation-tolerant (Figs. 23 and 24). The beginning of sporulation was indicated by a thickened plasmalemma and the development of a more completely organized endoplasmic reticulum in the early secondary sporonts, which occurred in chains (Figs. 25 and 26). All stages in the sporulation sequence were diplokarytic (Figs. 23–27) and the nuclei contained distinct nucleoli (Fig. 25).

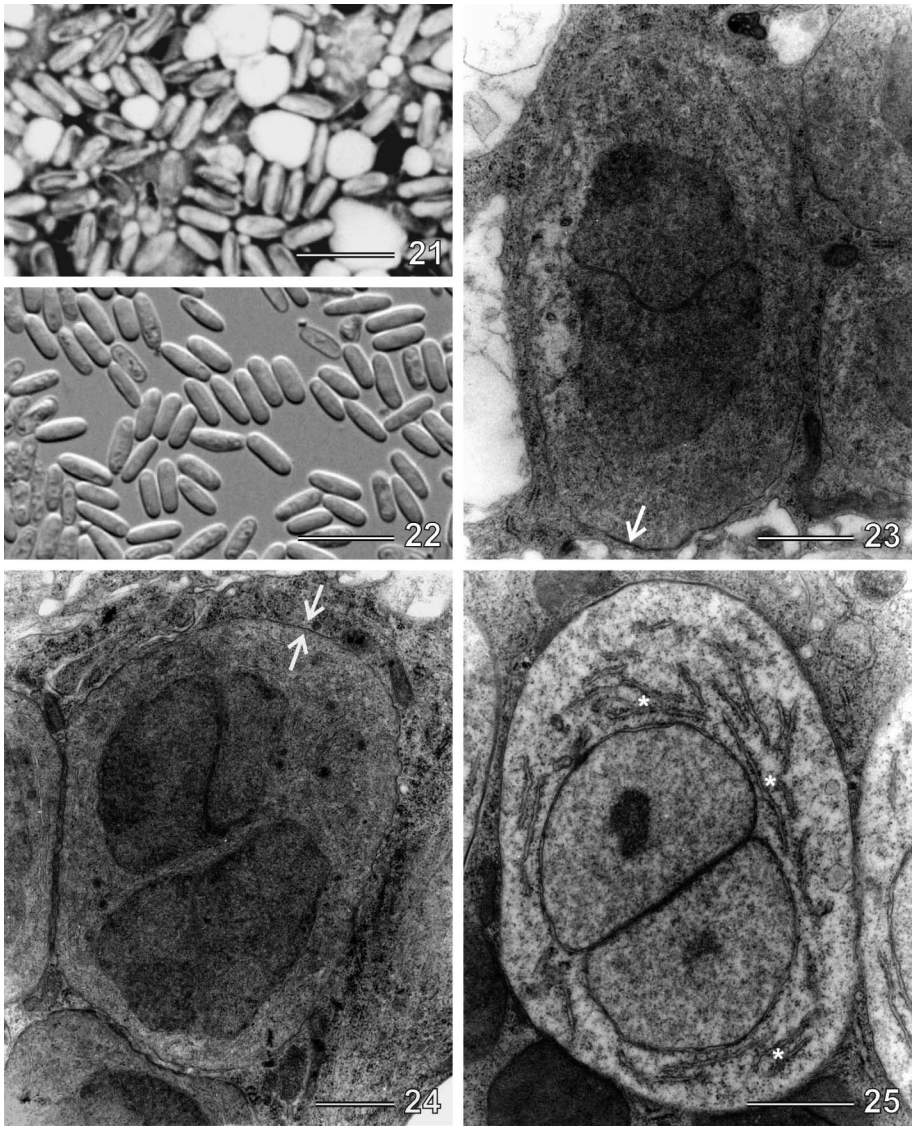
Ultrastructural observation of the secondary spores confirmed the presence of two nuclei in diplokaryotic arrangement and 10–12 coils of the isofilar polar filament (Figs. 28–30). The polaroplast was typical for microsporidia of the *Nosema*-type consisting of tight lamellae anteriorly and irregular lamellae posteriorly. At the anterior pole of the spore was the mushroom shaped anchoring disc and the manubroid portion of the polar filament (Fig. 28). The exospore appeared to be relatively thick (46–58 nm), with a single, somewhat wavy layer of material of medium electron density. The endospore of the secondary spores was thicker than that of the primary spores (81–93 nm vs. 70–75 nm) and was revealed as single layer of material of low electron density (Figs. 28–30).

3.4. Infected tissues, pathogeny

Midgut epithelial and muscle cells, and adhering hemocytes, were found infected at 24 h after ingestion of infec-



Figs. 14–20. *Nosema chrysorrhoeae*, secondary developmental stages as seen in Giemsa-stained smears from salivary glands of the *Euproctis chrysorrhoea* Figs. 14 and 15. Binucleate and tetranucleate meronts. Figs. 16–19. Sporonts of the secondary developmental cycle undergoing gradually the disporoblastic sporogony. Fig. 20. Nuclear division of the sporont with characteristically located nuclei on cell poles gives rise by cytokinesis to two sporoblasts. (Bars: Figs. 14–19 = 5 μm, Fig. 20 = 10 μm.)



Figs. 21–25. Light micrographs of secondary spores and transmission electron micrographs of the secondary developmental cycle of *Nosema chrysorrhoeae*. Figs. 21 and 22. Secondary spores: Giemsa stained dry smear (Fig. 21), fresh spores in Nomarski interference contrast (Fig. 22). Note that the spores do change shape on drying and staining. (Bars = 10 μm.). Figs. 23 and 24. Binucleate (Fig. 23) and tetranucleate (Fig. 24) early sporonts. Densifications of the plasma membrane characterizing sporogony are at arrows. Note the nearly absence of the endoplasmic reticulum. (Bars = 1 μm.). Fig. 25. More advanced sporont showing the increasing numer of the endoplasmic reticulum lamellae (∗). (Bar = 1 μm.)

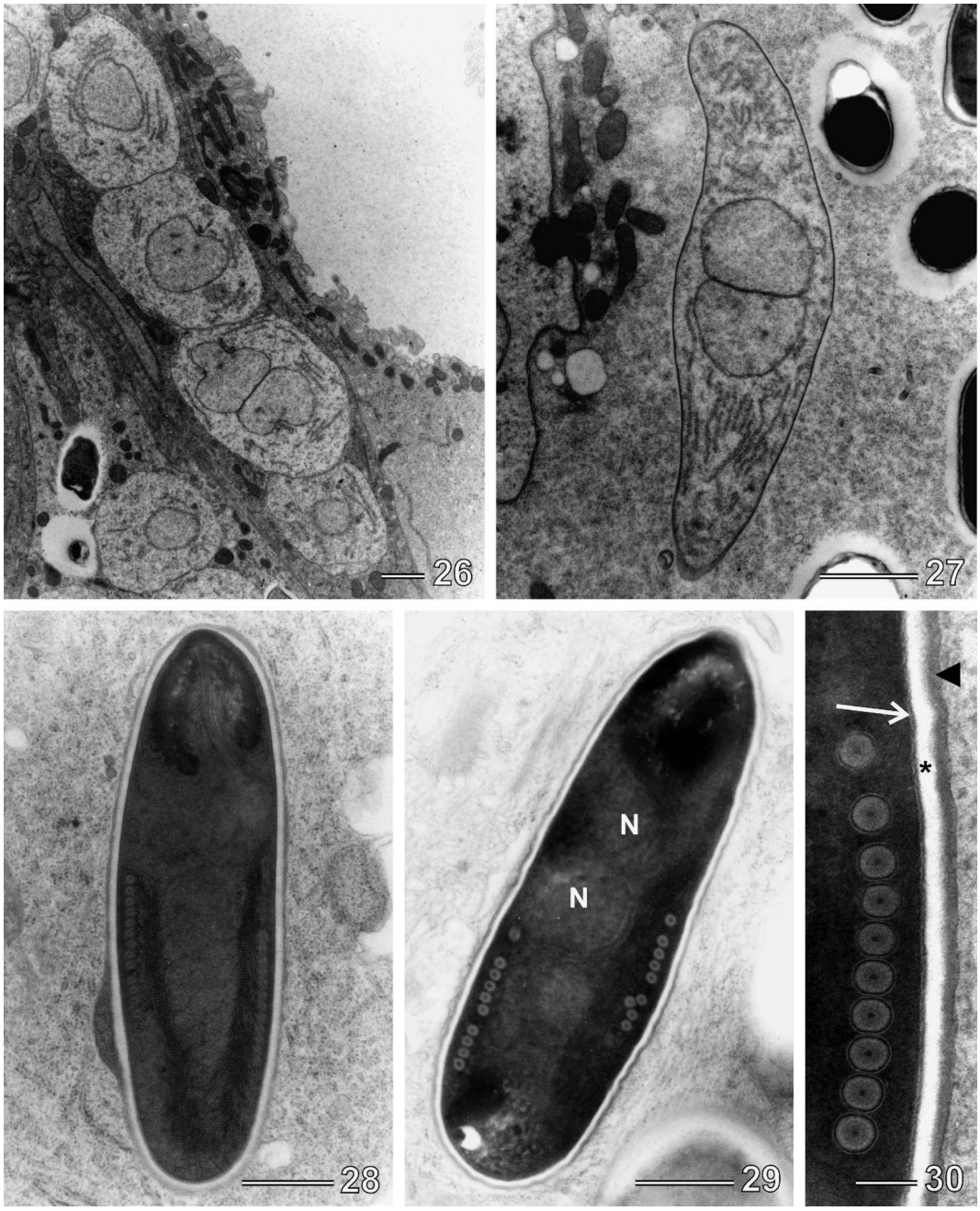
tive secondary spores, and a small number primary spores were also detected in the Malpighian tubules. In these tissues, primary spores appeared 2 dpi and proliferated until 6 dpi. Mature secondary spores were first observed 7 dpi and were located exclusively in the silk glands, which became heavily infected 10 dpi. No host mortality was observed in the laboratory. Infected larvae were similar size to uninfected controls and pupated normally.

3.5. Host specificity

All tested laboratory hosts, *L. dispar*, *M. brassicae*, and *Spodoptera littoralis*, were refractory; no parasite stages were found in these hosts when dissected 7–12 dpi.

3.6. Phylogenetic relationship

The sequenced region of SSU rDNA in *N. chrysorrhoeae* differs from that of *Nosema portugal*, *Nosema vespula* and *Vairimorpha lymantriae* Leviste (a lymantriid *Nosema*-type isolate) in five unique nucleotide positions. When compared to *Vairimorpha lymantriae* AF033315, *N. chrysorrhoeae* SSU rDNA region possesses six unique nucleotide positions. There are overall seven different nucleotide positions in the sequenced region of ITS and LSU rDNA among *N. chrysorrhoeae*, *N. kovacevici*, *N. serbica*, *V. lymantriae* isolates and *Nosema* sp. from *L. monacha*. However, four positions are unique to a single species and only three have parsimony informative character: in the internal transcribed spacer region at position 1258, in the large sub-



Figs. 26–30. Presporal stages and spores of the secondary cycle of *Nosema chrysorrhoeae* Fig. 26. Chain of sporonts. Fig. 27. Binucleate cell at the sporont/sporoblast transition stage. Figs. 28 and 29. Secondary binucleate (N) spores. Fig. 30. Detail of the secondary spore showing 10 isofilar coils of the polar filament arranged in one row, the spore plasma membrane (arrow), relatively thick translucent endospore (∗) and relatively thick, but structurless exospore (arrowhead). (Bars: Figs. 26–29 = 1 μm, Fig. 30 = 0.25 μm.)

unit rDNA region at position 1481 and at position 1530 (Table 1).

All trees obtained using maximum parsimony (MP) and maximum likelihood (ML) as inferred from SSU rRNA as well as LSU rRNA sequence data are nearly identical, and clearly support the inclusion of *Nosema chrysorrhoeae* within the clade of lymantriid *Nosema*/*Vairimorpha* as a separate taxon. The maximum likelihood tree (Fig. 31) based on SSU rRNA gene sequences shows that the *Nosema*/*Vairimorpha* clade consists of two highly supported subclades (B,C) with *N. chrysorrhoeae* belonging to the "hymenopteran subgroup" (B), as it is defined according to the frame and preponderance of hymenopteran microsporidia. On more detailed topology trees *N. chrysorrhoeae* with 95 ML-bootstrap support (Fig. 31 tree B) forms a group both with the microsporidians parasiting another lymantriid host *Lymantria dispar* (*N. portugal* and *V. lymantriae*) and the hymenopteran microsporidium *Nosema vespula*.

The maximum parsimony tree (Fig. 32) based on partial LSU rRNA gene sequences confirms the crown (most

Table 1
Differences in respective nucleotide positions in the ITS and lsu rDNA region

	1258[a] #	1375#	1481[a]	1530[a]	1630	1666	1677
N. chrysorrhoeae n. sp.	T	A	C	T	G	T	G
N. kovacevici	T	A	C	T	T	T	G
N. portugal	T	T	ND	ND	ND	ND	ND
N. serbica	C	A	C	T	G	T	A
N. sp. (*L. monacha*)	C	A	C	A	G	—	G
V. lymantriae coll.	C	A	A	A	G	T	G

[a] Parsimony informative positions, # ITS region, ND not done.

derived) position of microsporidia of the *Nosema*/*Vairimorpha* clade as described in the trees based on SSU rRNA gene sequences. Similarly to that, the *Nosema*/*Vairimorpha* clade is composed of two main subgroups: the group around *Nosema bombycis* and the "hymenopteran subgroup." The "hymenopteran subgroup" contains another two distinct subgroups: the first one includes *Nosema apis* and closely related species around the *Vairimorpha necatrix*, while the second is composed of species isolated from the lymantriid host: *Nosema* sp. from *L. monacha*, *N. serbica*, and *V. lymantriae* from the gypsy moth (*Lymantria dispar* L.) and *N. kovacevici* and *N. chrysorrhoeae* from the browntail moth (*E. chrysorrhoea* L.).

4. Discussion

4.1. Assignment to the genus Nosema and comparison with Nosema species from E. chrysorrhoea and other lymantriids

The life cycle of the species we describe herein as *Nosema chrysorrhoeae* n. sp. is typical for microsporidia of the genus *Nosema* (Iwano and Kurtti, 1995; Solter and Maddox, 1998) and is very similar to the cycle of *Nosema portugal* as described by Maddox et al. (1999). Several reports exist of *Nosema* species, which differ in host tissue specificity, spore shape, and size, found in *E. chrysorrhoea* (Table 2).

Nosema chrysorrhoea possesses several characteristics that distinguish it from *N. kovacevici* and other *E. chrysorrhoea* microsporidian isolates. *N. chrysorrhoeae* produces mature secondary spores that are uniform, more elongate and spindle-shaped. In addition, the secondary developmental cycle occurs exclusively in the silk glands of the host.

The *E. chrysorrhoea* microsporidian described here is related to other *Nosema* spp. parasitizing the closely related host, *L. dispar*: *Nosema muscularis* Weiser, 1957; *Nosema lymantriae* Weiser, 1957; *Nosema serbica* Weiser, 1963 and *Nosema portugal* Maddox and Vávra, 1999. *Nosema muscularis*, found in midgut epithelium and midgut muscles, is presently considered to be the primary spore form of some *Nosema* or *Vairimorpha* microsporidia (Weiser and Linde, 1998). Several microsporidia of the genera *Nosema* and *Vairimorpha* produce primary spores of *Nosema muscularis*-type. We belive that *Nosema muscularis* is a junior synonym of *Vairimorpha disparis* (Vávra et al., 2006). *Nosema lymantriae* infects the fat body and silk glands of its host. Both *N. serbica* Weiser, 1963 and *N. portugal* Maddox and Vávra, 1999 differ from *N. chrysorrhoeae* in tissue specificity, spore size and shape, and the number of polar filament coils (Pilarska and Vávra, 1991; Maddox et al., 1999). Both infect fat body tissues. In addition, *Nosema portugal* possesses polar filament-like tubules in secondary spores.

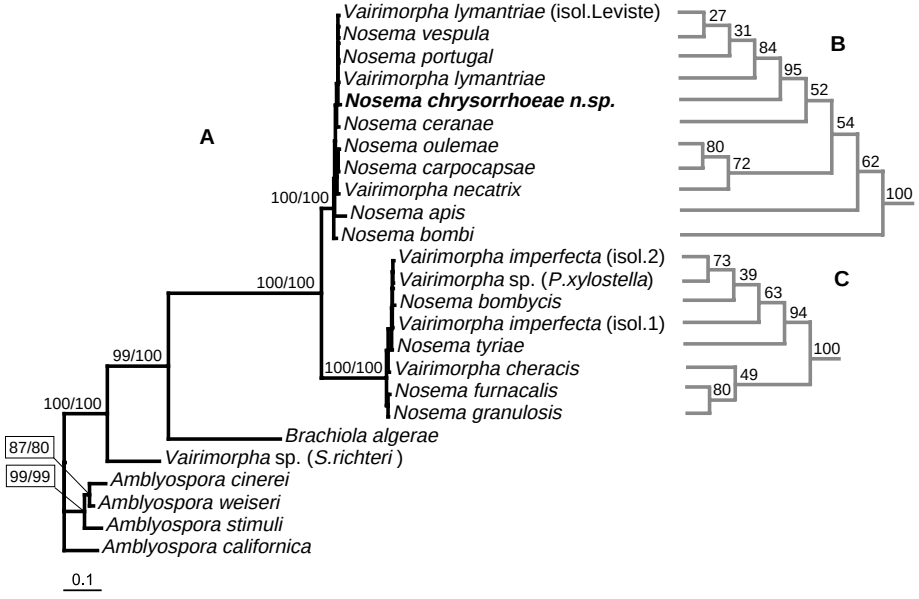


Fig. 31. A. Maximum likelihood (ML) phylogenetic tree (log lk = −3810.62976) as inferred from partial SSU rRNA gene sequences. Tree was constructed using PhyML program with general time reversible (GTR) model for nucleotide substitutions and discrete gamma distribution in 8 + 1 categories. All parameters (gamma shape = 0.598; pinvar = 0.014) were estimated from dataset. Numbers above branches indicate ML-bootstrap support (HKY 85 model, one category of sites, tv/ti rate estimated from dataset, 500 replicates)/maximum parsimony (MP) bootstrap support (1000 replicates). B and C show detailed topologies of particular clusters together with ML-bootstrap support computed as mentioned.

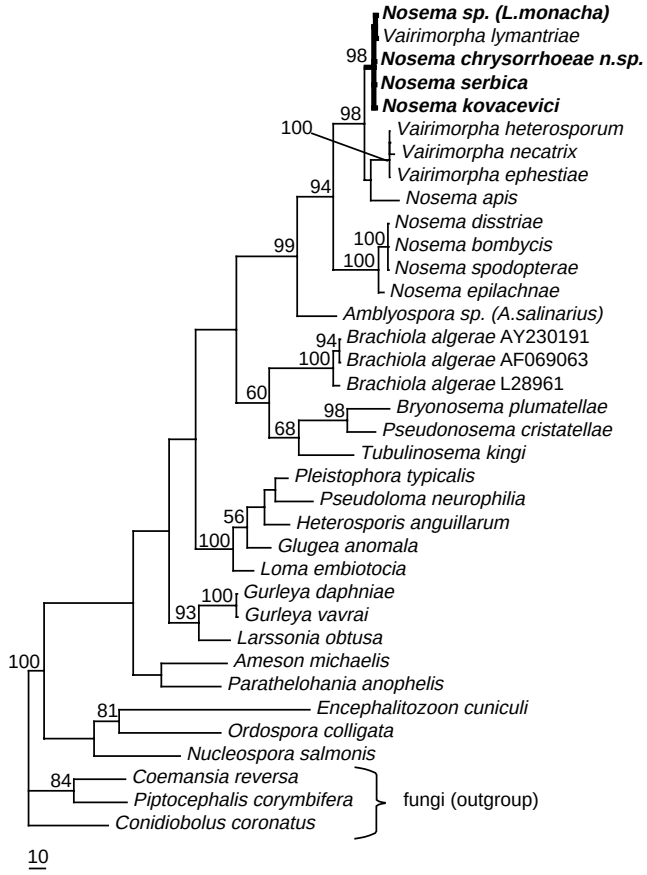


Fig. 32. One of 30 equally parsimonious trees as inferred from partial LSU rRNA gene sequences (positions 1–580 in *Escherichia coli*) Alignment contained 219 characters, 177 characters were parsimony informative. Tree is 1156 steps long, CI = 0.4118; RI = 0.6777. Numbers above branches indicate maximum parsimony bootstrap support (1000 replicates).

Nosema chrysorrhoeae appears to be specific to the host, *E. chrysorrhoea*. Host specificity is an important character in the consideration of closely related lepidopteran microsporidia (Solter et al., 1997; Linde et al., 1998). It is a distinguishing character of the newly described microsporidium. *N. chrysorrhoeae* is not infective to *Mamestra brassicae*, *Spodoptera littoralis*, or the closely related *L. dispar*, nor is *N. lymantriae* from *L. dispar* infective to *E. chrysorrhoea* (Weiser, 1957).

Nosema chrysorrhoeae falls within the lymantriid *Vairimorpha*/*Nosema* clade based on SSU rDNA and LSU rDNA sequencing (Figs. 31 and 32). This clade is a sister group to the *V. necatrix* clade, and is less related to the *Nosema bombycis* group. Vossbrinck and Friedman (1989), Preparata et al. (1989) and Baker et al. (1994) demonstrated that sequence data from the 580R region of LSU rDNA is an appropriate character to evaluate relationships at or near the species level. Isolates that differ at one or more nucleotides in the 580R region have been found, in all cases, to be valid species. Our data support this finding and, combined with biological and structural characters we report, the designation *N. chrysorrhoeae* is justified.

Taxonomic summary:

Systematics: *Nosema chrysorrhoea* n. sp. M. Hyliš and D.K. Pilarska

Type host: Browntail moth, *Euproctis chrysorrhoea* L.

Transmission: Per os

Site of infection: *Primary developmental cycle: midgut epithelial and muscle cells, hemocytes. Secondary developmental cycle: silk glands*

Interface: *All stages in direct contact with host cell cytoplasm*

Development: Two types of spores formed in the host in primary and secondary sporogonic cycle. Diplokaryotic in all life stages; meiosis not observed. Binucleate and tetranucleate meronts. Sporogony disporoblastic. Thin-walled ovoid primary spores with large posterior vacuole measured 4.1 (4.5–5.2) × 1.9 (2.1–2.2) μm (fixed, stained), have electron dense, single layer, smooth exospore (25–30 nm) and thin, lucent endospore (70–75 nm), 5–6 isofilar polar filament coils. Thick-walled long ellipsoidal to spindle-shaped secondary spores measured 6.12 (5.52–6.67) × 2.21 (1.99–2.38) μm (fresh), have 46–58 nm thick exospore with a single wavy layer of material of medium electron density and 81–93 nm thick endospore revealed as single layer of material of low electron density, 10–12 isofilar polar filament coils. Polaroplast with the tight lamellae anteriorly, irregular posteriorly.

Type locality: *Vicinity of Karlovo*, *Central Bulgaria*, *host on Q. cerris*

Remarks: Partial nucleotide sequences, SSU, ITS and LSU rDNA are deposited in the NCBI GenBank, Acces-

Table 2
Nosema spp. reported from *Euproctis chrysorrhoea* L

	Spore size, shape, number of polar filament coils	Site of infection	Lokation	Reference
Nosema sp.	4.5–8.2 × 2.0–3.5 μm; vary in shape and size	General infection, mostly fat body and silk glands	Poland	Lipa (1964)
Nosema kovacevici	2.5–6 × 2.0–2.3 μm; characteristic variability in length; elongate	Not given	Yugoslavia—Kosovo	Purrini and Weiser (1975)
Nosema sp.	4.2 × 2.6 μm; oval	Fat body, midgut, hypodermis, hemocytes	Yugoslavia—Macedonia	Sidor et al. (1975)
Nosema sp.	5–6 × 2–2.5 μm; oval; 11–14	General infection	Bulgaria	Pilarska and Weiser, unpublished
Nosema sp.	3.38–5.45 × 1.48–2.28 μm; oval; 9–10	Fat body and silk glands	Bulgaria	Pilarska et al. (2002)
Nosema chrysorrhoeae n. sp.	5.52–6.67 × 1.99–2.38 μm; elongate, spindle shape; 10–12	Silk glands	Bulgaria	Present study

sion Nos. AY940656 and AY940657. Syntype slides, Giemsa stained smears of infected silk glands of E. chrysorrhoea, labeled "*N. chrysorrhoeae*" are placed in the type slide collection of Dr. Jaroslav Weiser, Prague, Czech Republic and the Illinois Natural History Survey, Urbana, USA.

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